characteristic of those made by both *B. thuringiensis kurstaki* and *B. thuringiensis tenebrionis*. U. S. Patent 4,910,016 (corresponding to EP 0303379) discloses a *B. thuringiensis* isolate identified as *B. thuringiensis* MT 104 which has insecticidal activity against coleopterans and lepidopterans.

1.2.4 CRY1 ENDOTOXINS

The characterization of the lepidopteran-toxic *B. thuringiensis* Cry1Aa crystal protein, and the cloning, DNA sequencing, and expression of the gene which encodes it have been described (Schnepf and Whitely, 1981; Schnepf *et al.*, 1985). In related publications, U. S. Patent 4,448,885 and U. S. Patent 4,467,036 (specifically incorporated herein by reference), the expression of the native *B. thuringiensis* Cry1Aa crystal protein in *E. coli* is disclosed.

Several *cry1C* genes have been described in the prior art. A *cry1C* gene truncated at the 3' end was isolated from *B. thuringiensis* subsp. *aizawai* 7.29 by Sanchis et al. (1988). The truncated protein exhibited toxicity towards Spodoptera species. The sequence of the truncated *cry1C* gene and its encoded protein was disclosed in PCT WO 88/09812 and in Sanchis *et al.*, (1989). The sequence of a *cry1C* gene isolated from *B. thuringiensis* subsp. *entomocidus* 60.5 was described by Honee *et al.*, (1988). This gene is recognized as the holotype *cry1C* gene by Höfte and Whiteley (1989). The sequence of a *cry1C* gene is also described in U. S. Patent 5,126,133.

The *cry1C* gene from *B. thuringiensis* subsp. *aizawai* EG6346, contained on plasmids pEG315 and pEG916 described herein, encodes a Cry1C protein identical to that described in the aforementioned U. S. Patent 5,126,133. The Cry1C protein described by Sanchis *et al.*, (1989) and in PCT WO 88/09812 differs from the EG6346 Cry1C protein at several positions that can be described as substitutions within the EG6346 Cry1C protein: Cry1C N366I, W376C, P377Q, A378R, P379H, P380H, V386G, R775A.

Significantly, the amino acid positions 376-380 correspond to amino acid residues predicted to lie within the loop region between β strand 6 and β strand 7 of Cry1C, using the nomenclature adopted by Li *et al.* (1991) for identifying structures within Cry3A.

Bioassay comparisons between the Cry1C protein of strain EG6346 and the Cry1C protein of strain *aizawai* 7.29 revealed no significant differences in insecticidal activity towards *S. exigua*, *T. ni*, or *P. xylostella*. These results suggested that the two Cry1C proteins exhibited the same insecticidal specificity in spite of their different amino acid sequences within the predicted loop region between β strand 6 and β strand 7.

Smith and Ellar (1994) reported the cloning of a *cry1C* gene from *B. thuringiensis* strain HD229 and demonstrated that amino acid substitutions within the putative loop region between β strand 6 and β strand 7 ("loop β 6-7") altered the insecticidal specificity of Cry1C towards *Spodoptera frugiperda* and *Aedes aegypti* but did not improve the toxicity of Cry1C towards either insect pest. These results appeared to conflict with the aforementioned bioassay comparison between the EG6346 Cry1C protein and the *aizawai* 7.29 Cry1C protein showing no effect of amino acid substitutions within loop β 6-7 of Cry1C on insecticidal specificity. Accordingly, the *cry1C* gene from strain *aizawai* 7.29 was re-sequenced where variant codons for the active toxin region were reported by Sanchis *et al.*, (1989) and in PCT WO 88/09812. The results of that sequence analysis revealed no differences in the amino acid sequences of the active toxins of Cry1C from strain EG6346 and of Cry1C from strain *aizawai* 7.29. Thus, the prior art on the Cry1C protein of strain *aizawai* 7.29, in light of the aforementioned bioassay comparisons with the Cry1C protein of strain EG6346, incorrectly taught that multiple amino acid substitutions within loop β 6-7 of Cry1C had no effect on insecticidal specificity. Recently, Smith *et al.*, (1996) also reported unspecified sequencing errors in the *aizawai* 7.29 *cry1C* gene.

1.2.5 Molecular Genetic Techniques Facilitate Protein Engineering

The revolution in molecular genetics over the past decade has facilitated a logical and orderly approach to engineering proteins with improved properties. Site specific and random mutagenesis methods, the advent of polymerase chain reaction (PCR™) methodologies, and related advances in the field have permitted an extensive collection of tools for changing both amino acid sequence, and underlying genetic sequences for a variety of proteins of commercial, medical, and agricultural interest.

Following the rapid increase in the number and types of crystal proteins which have been identified in the past decade, researchers began to theorize about using such techniques to improve the insecticidal activity of various crystal proteins. In theory, improvements to δ-endotoxins should be possible using the methods available to protein engineers working in the art, and it was logical to assume that it would be possible to isolate improved variants of the wild-type crystal proteins isolated to date.. By strengthening one or more of the aforementioned steps in the mode of action of the toxin, improved molecules should provide enhanced activity, and therefore, represent a breakthrough in the field. If specific amino acid residues on the protein are identified to be responsible for a specific step in the mode of action, then these residues can be targeted for mutagenesis to improve performance.

1.2.6 STRUCTURAL ANALYSES OF CRYSTAL PROTEINS

The combination of structural analyses of *B. thuringiensis* toxins followed by an investigation of the function of such structures, motifs, and the like has taught that specific regions of crystal protein endotoxins are, in a general way, responsible for particular functions.

For example, the structure of Cry3A (Li *et al.*, 1991) and Cry1Aa (Grochulski *et al.*, 1995) illustrated that the Cry1 and Cry3 δ-endotoxins have three distinct domains. Each of these domains has, to some degree, been experimentally determined to assist in a particular function. Domain 1, for example, from Cry3B2 and Cry1Ac has been found to be responsible for ion channel activity, the initial step in formation of a pore (Walters *et al.*, 1993; Von Tersch *et al.*, 1994). Domains 2 and 3 have been found to be responsible for receptor binding and insecticidal specificity (Aronson *et al.*, 1995; Caramori *et al.*, 1991; Chen *et al.* 1993; de Maagd *et al.*, 1996; Ge *et al.*, 1991; Lee *et al.*, 1992; Lee *et al.*, 1995; Lu *et al.*, 1994; Smedley and Ellar, 1996; Smith and Ellar, 1994; Rajamohan *et al.*, 1995; Rajamohan *et al.*, 1996; Wu and Dean, 1996). Regions in domain 3 can also impact the ion channel activity of some toxins (Chen *et al.*, 1993, Wolfersberger *et al.*, 1996).